

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/89, 15/85, C12P 21/00, A61K 48/00	A1	(11) International Publication Number: WO 97/11191 (43) International Publication Date: 27 March 1997 (27.03.97)
(21) International Application Number: PCT/US96/15083 (22) International Filing Date: 20 September 1996 (20.09.96) (30) Priority Data: 60/004,115 21 September 1995 (21.09.95) US (71)(72) Applicant and Inventor: MILLER, Ira [US/US]; 46 Blake Street, Newton, MA 02160 (US). (74) Agents: WILDES, Morey, B. et al.; Cowan, Liebowitz & Latman, P.C., 1133 Avenue of the Americas, New York, NY 10036-6799 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHOD OF TRANSGENE EXPRESSION AND SECRETION IN SCHISTOSOMA		
(57) Abstract This invention is a method of producing schistosomes as an intermediate transgene vector for secretion of desired gene products. The desired products are secreted into the bloodstream of the host (patient) by schistosomes that have been engineered through the germline with DNA encoding the transgene. The use of schistosomes as an intermediate vector facilitates mass production, quality control, termination of therapy at will and dose titration. The method is applicable to situations in which the acquired protein is functional in the plasma or in endocytotic vesicles.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

METHOD OF TRANSGENE EXPRESSION AND SECRETION IN SCHISTOSOMA**BACKGROUND**

This invention relates to the field of gene therapy. Most current strategies of gene therapy employ mechanisms to alter the patient's own cells to produce the desired gene product, through viral and non-viral vectors that introduce DNA that encodes the desired product. Some of the major pitfalls of such methods are: low efficiency of introduction and expression, potential for viral infection by contaminating replication competent virus, potential for recombination with host DNA and for promoting malignant transformation, irreversibility of the process, and the need for labor-intensive individualized treatment. The method described here avoids these problems because it uses an intermediate vector for gene expression in the patient, a vector that can be mass-produced and batch-characterized, that can be eliminated at will and that does not alter the DNA of the patient's own cells.

This patent describes a method of creating genetically engineered schistosomes as a vector for secretion of therapeutic proteins into the bloodstream of humans and other susceptible hosts. This process will result in a sustained *in vivo* protein expression system. This system avoids the need for large scale protein purification and for repeated injections of therapeutic proteins that must be administered parenterally, such as insulin or erythropoietin. This mode of protein expression is a form of "gene therapy" applicable in situations where the gene introduced, hereafter referred to as the "transgene", does not require expression in the cells of the patient but rather can be functionally expressed in an intermediate, symbiotic vector.

Humans are hosts to seven schistosome species, the blood flukes (Rollinson D and Southgate VR, *The Genus Schistosoma: A Taxonomic Appraisal*. pp. 1-49 in D. Rollinson, AJG Simpson, Eds., *The Biology of Schistosomes from Genes to Latrines*, London Academic Press, 1987, reviewed in Basch PF, Overview, pp. 3-33, in *Schistosomes: Development, Reproduction, and Host Relations*, PF Basch, N w York, Oxford University Press, Inc.,

1991). Schistosomes are parasites endemic in many third world countries where poor sanitation and manual farming practices allow perpetuation of the species through their various life cycle stages. These stages include the sporocyst forms that grow in fresh water snails and the adult worms that develop and mate within the human blood stream. The adult worms lay eggs in the wall of the bladder or intestine, most of which are eventually expelled through the stool or the urine. The mechanisms that allow the adult worms to evade the human host's immune response and to survive in the bloodstream for many years without causing symptoms are not completely understood. However, this ability makes them a useful vector for delivery of therapeutic proteins into the human bloodstream. Infection with schistosomes is not innocuous, though, for, in individuals who harbor many parasites, a characteristic disease ensues caused by the body's response to eggs that fail to get expelled. The retained eggs flow through the veins and provoke a granulomatous response leading to fibrosis of tissues where they are deposited: the liver, for species that live in the portal veins (e.g. *S. mansoni*), or the bladder wall, for species that live in the pelvis (e.g. *S. haematobium*). To avoid this problem in recipients of the schistosome vector for protein expression, this patent deals with the creation of transgenic schistosomes which lay soft, degradable eggs or which lay eggs with reduced sclerotin content.

It is desirable to provide a wide variety of possible transgene products for insertion into the vector. An exhaustive list of potential products for expression in this system is not intended. Theoretically, the system described herein is suitable for expression of any protein that is active in the plasma or that can be targeted from the bloodstream to its appropriate extracellular or intracellular location. Several categories of proteins suitable for this therapeutic system are noted below, with examples given for each.

It is desirable to provide for the insertion of protein and glycoprotein hormones, such as:

- insulin: Although blood-glucose-level-regulated-expression of insulin is required for proper glucose control,

a constant, low-level baseline expression of insulin may prove to be extremely valuable for preventing hyperglycemic episodes leading to ketoacidosis. In addition, low level baseline expression may reduce the number of daily injections needed
5 for many insulin-dependent diabetics.

- leptin: This newly discovered adipocyte hormone is sure to play a role in body fat regulation for many individuals. Probably, only low levels of expression are required for therapeutic benefit, and it does not require a
10 timed expression pattern.

- calcitonin: Osteoporosis is a major cause of morbidity and mortality among post-menopausal women, the elderly and steroid-dependent individuals. Calcitonin injections are one mode of therapy used for such individuals. A boost in
15 baseline calcitonin levels using this vector may replace that mode of therapy.

It is also desirable to provide for the insertion of non-hormonal circulating proteins, such as:

- alpha-1-anti-trypsin: Deficiency of this plasma
20 protein causes significant disease in 1/3500 individuals, leading to cirrhosis as well as emphysema. Constant low levels of expression are required to prevent tissue destruction.

- factor VIII: Deficiency of this protein causes
25 hemophilia in 1/10,000 males. Constant low serum levels are required to prevent morbidity.

- cholesterol ester transfer protein inhibitor: Aberrant lipoprotein profiles are a significant cause of morbidity from atherosclerosis. Agents acting to increase
30 HDL/LDL cholesterol, such as a peptide designed to inhibit this enzyme, may have a tremendous effect on disease in individuals at risk.

- human immunodeficiency virus co-receptor ligands: Recently, co-receptors for HIV on T-cells (the SDF-1 chemokine
35 receptor, LESTR/fusin) and macrophages (the beta-chemokine receptor, CC-CKR) have been identified. Individuals with elevated levels of beta-chemokines are resistant to HIV infection. Artificially raising serum levels of the ligands

for these receptors may protect against infection with HIV or slow disease progression.

In addition, it is desirable for this method to be used against lysosomal storage diseases, for example, for the insertion of beta-glucocerebrosidase, deficiency of which causes Gaucher's disease, most common in Ashkenazic Jews. Exogenous administration of the purified enzyme from placenta is potentially curative, as the protein is targeted to the lysosomal compartment. Therapy is presently limited by availability of enzyme, which must be repetitively injected.

It is further desirable to combat borderline deficiency protein states contributory to disease, for example, with regard to deficiency of CRI. In collagen vascular diseases triggered by immune complexes (e.g. SLE), erythrocyte complement receptor levels are reduced, leading to delayed clearance of circulating immune complexes and deposition in tissues, with ensuing glomerulonephritis or vasculitis. Expression of a soluble CRI receptor might facilitate clearance of immune complexes by the reticuloendothelial system and prevent relapses.

Furthermore, many diseases are caused by defects or deficiencies of intracellular non-lysosomal (e.g. cytosolic, mitochondrial) enzymes or structural proteins. Methods may be developed in the future to allow postranslational transmembrane passage of desired proteins, possibly based upon the paradigm of the dimeric ricin and diphtheria toxins. Then the schistosome expression vector could be used to deliver proteins to treat glycogen storage diseases, hormone receptor defects, and many metabolic disorders requiring replacement of a cytosolic or even possibly subcellularly localized (not only lysosomal) protein.

Moreover, proposals for therapy based upon novel protein constructs herald the next phase of therapeutic advances such as expression of novel inhibitor proteins, hybrid proteins, magic bullet proteins, etc. These ideas include combinations of antibody variable region domains and toxins to kill cells bearing specific antigens, juxtaposition of antigens with negatively signaling molecules to induce tolerance, and dummy viral receptors (e.g. CD4, to fight HIV.)

SUMMARY OF THE INVENTION

In general, the invention is a transgenic schistosome, male or female depending on the particular embodiment, whose genome has been stably transformed by DNA encoding a transgene within appropriate regulatory contexts. The transgenic schistosome secretes the transgene product into the bloodstream of its human or other definitive host. The invention takes advantage of developed methods for propagating schistosomes in snails at the sporocyst life cycle stage in order to obtain large clonal populations of recombinant schistosomes. The method adapts existing technology that has been developed for microinjection of eggs of other species for use in the injection of schistosome eggs, based upon known aspects of schistosome biology.

The invention includes methods to use male schistosomes as vectors in unisexual infections and to use female schistosomes in bisexual infections. In female infections, methods to decrease egg production and eggshell maturation are described. The invention uses specific types of DNA constructs encoding antisense RNAs and ribozymes to interfere with schistosome eggshell protein production or maturation either directly or by interfering with the action of tissue-specific transcription factors, and the invention describes methods to clone these transcription factors and eggshell tanning enzymes. The invention employs use of schistosome genomic DNA locus control region-like elements to confer high level tissue-specific expression of the transgene, based upon work done in the mouse, and it describes how to identify and utilize these regulatory regions for creation of the transgene construct. The invention utilizes DNA constructs encoding various mRNA regulatory sequences and signal peptides based upon published schistosome and non-schistosome sequences. The invention describes strategies for mating recombinant schistosomes to obtain the most effective transgenic schistosome vector.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow-chart of the method for obtaining clones of recombinant schistosome clones;

Figure 2 is a diagrammatic representation of how to adapt endogenous schistosome gene transcriptional regulatory sequences for use in the transgene vector;

Figure 3 is a diagrammatic flow-chart of how to obtain female transgenic clones on an eggshell knockout background; and

Figure 4 is a diagram of the transcriptional regulatory regions to accompany the various transcripts of the multiple DNA constructs to be used in 2-step and 3-step schistosome genetic modifications.

DETAILED DESCRIPTION OF THE INVENTION

I. General Considerations:

One way to use schistosomes as expression vectors without causing granulomatous disease is to treat patients with only male worms, so that no granuloma-promoting eggs are laid. In species that infect humans, the male is more robust than the female, which is dependent upon the male for transport through the venous plexuses that they inhabit (Standen OD, The Relationship of Sex in *Schistosoma mansoni* to Migration Within The Hepatic Portal System of Experimentally Infected Mice, Annals of Tropical Medicine and Parasitology 47:139-145, 1953, reviewed in Basch PF, Sexual and Conjugal Biology, pp.137-171, Basch PF, 1991). However, male worms of some species mature in the absence of females (*S. Mansoni*; Vogel H, 1941 Infektions-versuche an verschiedenen Bilharzia-Zwischenwirten mit einem einzelnen Mirazidium von *Bilharzia mansoni* und *B. japonica* Zentrallblatt fur Bakteriologie Abteilung I. Originale 148: 29-35, *S. Haematobium*; Sahba GH and Malek EA, 1977 Unisexual infections with *Schistosoma haematobium* in the mouse, American Journal of Tropical Medicine and Hygiene 26:331-33, reviewed in Basch PF, 1991, Pp 137-71), and migrate almost completely to the appropriate location (i.e., the portal vein for *S. Mansoni*; Standen, 1953, and Armstrong JC, Mating Behavior and Development of Schistosomes in the Mouse, Journal of Parasitology 51:605-15, 1965) enabling use of male worms as vectors in unisexual infections.

In order to take advantage of male vectors in unisexual infections, the protein expression is targeted to the worm's

integument. Although the integument is not specialized for protein secretion, it nonetheless has a tremendous metabolic capacity for surface membrane protein production, a pathway to which exogenous proteins are targeted in this invention. The schistosome integument is a multi-laminate membrane (Silk MH et al., Ultrastructural studies of the blood fluke *Schistosoma mansoni* I, The integument, South African Journal of Medical Sciences 34:1-10, 1969; Hockley DJ and McLaren DJ, *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercaria to adult worm, International Journal for Parasitology 3:13-25, 1973; Torpier G, Capron M and Capron A, Structural changes of the tegumental membrane complex in relation to developmental stages of *Schistosoma mansoni* (Platyhelminthes: trematoda). Journal of Ultrastructural Research 61:309-24, 1977), that serves as a barrier to the host's immune system. Proteins targeted for secretion in this location should eventually find their way to the worm exterior, either after fusion of secretory vesicles with the exterior leaflet or after sloughing of the exterior leaflet, with release of material from the intermembranous space.

Transgene expression targeting to schistosome tissues other than the integument may, in fact, prove to be more efficacious. Structures that normally actively secrete soluble proteins are obvious targets for expression. These structures include the gut, the vitelline gland, the Mehlis gland and the ootype. In the gut, digestive enzymes (Gotz B and Klinkert M, Expression and partial characterization of a cathepsin B-like enzyme (Sm31) and a proposed 'haemoglobinase' (Sm32) from *Schistosoma mansoni*, Biochemistry Journal, 290:801-06, 1993) will most likely prevent significant amounts of intact exogenous protein from reaching the bloodstream, and any attempt to inactivate these enzymes will probably impair worm viability. The other structures mentioned are part of the female genital tract and are specialized for protein secretion, evidenced by their glandular structure and secretory epithelium, with ultrastructurally prominent Golgi apparatus and secretory vesicles (Spence IM and Silk MH, Ultrastructural studies of the blood fluke- *Schistosoma mansoni*: V, the female reproductive system- a preliminary

report, South African Journal of Medical Sciences, 36:41-50, 1971; Spence IM and Silk MH, Ultrastructural studies of the blood fluke- *Schistosoma mansoni* VI, the Mehlis gland, South African Journal of Medical Sciences, 36:41-50, 1971).

5 However, because of the female's dependence on contact with the male for maturation, targeted expression of exogenous proteins in these tissues of female worms requires bisexual infections in order to be utilized for human therapy, with the ensuing possibility of granuloma formation.

10 The unwanted side effect of egg production would be clinically relevant only if many worm pairs were to be required for many years, as only about 5-10% of infected individuals develop severe illness. For short term treatment, or if adequate levels of transgene product can be obtained
15 with only a few worm pairs, then egg laying may not be a significant clinical problem when weighed against the severity of the disease being treated.

In strategies targeting transgene expression to female organs, several methods are described to prevent release of
20 viable eggs in order to avoid causing granulomatous disease. One possible solution is, again, to use male-only infections, as some development of the female reproductive glands (vitelline or Mehlis glands) does take place even in male worms. These genotypically male partial hermaphrodites have
25 been found in unisexual male infections and never result in egg formation in schistosome species that infect humans (Shaw MK and Erasmus DA, *Schistosoma mansoni*: The Presence and Ultrastructure of Vitelline Cells in Adult Males, Journal of Helminthology 56:51-53, 1982; reviewed in Hermaphraditism in
30 Male Schistosomes, pp 162-164, in Ch. 4, Sexual and Conjugal Biology, in Schistosomes, PF Basch, 1991). Despite the possible use of hermaphrodite males, full utilization of the powerful secretory system of the female reproductive system most likely requires expression in females. For expression in
35 the most abundant and active female tissue, the vitelline gland, strategies are described below to interfere with the female's ability to make eggshell protein and enzymes involved in eggshell hardening.

II. General methods of schistosome propagation,
transformation and cloning

Isolates of schistosomes, including *S. mansoni*, *S. haematobium* and *S. japonicum* are obtained from stool (or
5 urine for *S. Haematobium*) from infected humans or from
established laboratory strains, and passaged in susceptible
Biomphalaria glabrata (or *Bulinus* for *S. Haematobium*) snails
and susceptible mouse strains or hamsters (see table 2-3 in
Basch, 1991, and references therein) as described (Hacket F,
10 The culture of *Schistosoma mansoni* and production of life
cycle stages, in *Methods in Molecular Biology*, vol. 21:
Protocols in Molecular Parasitology, JEH Hyde ed., Humana
Press Inc, Totowa New Jersey, 1993; MacInnis AJ, Maintenance
of *Schistosoma mansoni* and *Schistosomatium douthitti*, in
15 *Experiments and Techniques in Parasitology*, MacInnis A and
Voge M, Freeman Press, 1970; Smithers SR and Terry RJ, The
infection of laboratory hosts with cercaria of *Schistosoma
mansoni* and the recovery of the adult worms, *Parasitology*
55:695-700, 1965).

20 Schistosome eggs must first be isolated for microinjection.
The protocol for creating transgenic schistosomes is
shown in Figure 1. Freshly laid (stage I) eggs, containing
oocytes undergoing meiosis and male pronuclei, are harvested
by microdissection from the intestines of schistosome-infected
25 laboratory animals (Box 1), such as mouse, hamster or guinea
pig (Pellegrino et al., 1962, Pelligrino and Faria, 1965).
Infected animals are sacrificed in the week prior to the
forty-fourth day post-infection, when immature eggs
predominate (Pelligrino J et al., New approach to the
30 screening of drugs in experimental schistosomiasis mansoni in
mice, *American Journal of Tropical Medicine and Hygiene*
11:201-15, 1962; Pelligrino J and Faria J, The oogram method
for the screening of drugs in schistosomiasis mansoni,
American Journal of Tropical Medicine and Hygiene 14:363-69,
35 1965) and are identified in squash preparations of small
intestines as described (ibid, incorporated herein by
reference). Eggs in the same developmental stage are found in
clusters within a venule, and stage can be visually assessed
in a dissecting microscope based upon the cellularity of the

eggs (box 2) (ibid). Those in stage I are dissected away from intestinal tissue and placed sterily into a chamber for microinjection containing cell culture medium (see below) with 25 mM HEPES (pH 7.4) in place of bicarbonate. Collaginase treatment is performed at this point, if necessary, to remove adherent mouse tissue. Approximately 50 stage I eggs are removed from each maximally infected animal.

Older eggs have a dense eggshell coat that may prevent easy introduction of a microinjection needle. In addition, nuclear or cytoplasmic enzymes required for recombination may be present exclusively during early zygotic period. Identifying this early egg stage is also necessary because later stages, passed in the stool, contain multicellular developing miracidial organisms, injection of which may be more difficult and may lead to a non-clonal distribution of DNA integration in progeny, complicating the analysis of transgenics.

An alternative method for obtaining early stage eggs for microinjection is to isolate eight-week old adult schistosomes, culture them in vitro, and recover the earliest laid eggs from the culture medium as soon as possible. The technique for recovering adult schistosomes and the optimized parameters for culture of eggs to reach maturity in vitro have been assessed in detail (Newport G and Weller TH, 1982. Miricidia infective for snails derived from eggs laid by adult *Schistosoma mansoni* in vitro, Parasitology 84:481-90, 1982; Newport G and Weller TH, Deposition and maturation of eggs of *Schistosoma mansoni* in vitro: importance of fatty acids in serum-free media, American Journal of Tropical Medicine and Hygiene, 3:349-57, 1982; Wu GY et al., Stimulation of *Schistosoma mansoni* oviposition in vitro by animal and human portal serum, American Journal of Parasitology 74:618-22, 1982, all incorporated herein by reference).

Conditions for DNA microinjection (Box 3) are based upon optimized protocols from mouse egg microinjection (Brinster RL et al., Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs, Proceedings of the National Academy of Sciences, 82:4438-42, 1985), for which an integration efficiency of 27.1% has been achieved. The

schistosome zygotic nuclei are approximately 6 to 8 microns in diameter (Nez MM and Short RB, Gametogenesis in *Schistosomatium douthitti* (Cort) (Schistosomatidae: Trematoda), Journal of Parasitology 43: 167-82, 1957; Neill PJ et al.,

5 The ultrastructure of the *Schistosoma mansoni* egg, American Journal of Tropical Medicine and Hygiene 60: 429-30, 1988) slightly smaller than those of the mouse. Thus, up to one half the volume used by Brinster, up to 1pL of purified linear DNA with staggered ends are injected into the female or male

10 pronucleus at a concentration of 1-2ng/mcL (50-100 copies of a 10kb sequence) in 10mM TrisCl (pH 7.5) 0.25mM EDTA. DNA concentration, volume injected and presence of cytochalasin B (a mitotic inhibitor) in the egg incubation medium are varied to optimize egg survival and DNA integration efficiency in

15 initial experiments. Microinjection is performed with eggs placed under silicone oil on commercially-available depression slides or laboratory-prepared dried agarose coated glass cover slips, using standard techniques. Nuclear injection is confirmed in parallel control samples by using the

20 nondiffusible dye FITC-dextran to follow injections with fluorescence microscopy as has proven useful in microinjection of other helminths (Fire A, Integrative Transformation of *Caenorhabditis elegans*, The EMBO Journal 5:2673-80, 1986).

For miracidial maturation (Box 4) injected eggs are

25 maintained at 37°C in 5% CO₂ in bicarbonate buffered cell culture medium (see Newport and Weller, 1982, Parasitology, referenced above, for schistosome egg culture technique) with addition of fetal calf serum and casein hydrolysate for approximately six days, until the miracidia reach maturity.

30 Under the conditions reported, a workable proportion of the eggs reach maturity (approximately 12%) see table 1 (casein hydrolysate and mouse r.b.c.) see table 2 (8% fetal calf serum in DSMH), and their ability to productively infect snails was proven (ibid). Miracidial hatching and infection (Box 5) are

35 performed by placing individual mature eggs in small beakers containing a small amount of spring water and a single snail and exposing to bright light for five minutes as described (MacInnis AJ, 1970). Infected snails are then reared together until miracidia are produced (Box 6).

Propagation and analysis of recombinant clones (Boxes 7-11) is done preferably as follows: Because the released miracidia are used to infect susceptible snails on a one miracidium per snail basis, each snail releases thousands of genetically identical cercaria, which constitute a schistosomal clone. Analysis of infected snails is conducted weekly after the third week to determine which are productively infected. As described (McInnis AJ, 1970; Hyde JE, 1993, see above), snails, reared in darkness, are exposed to light for 60 minutes in deionized water, and the released cercaria are killed, stained with Lugol's iodine and counted microscopically in 10mcL aliquots (Box 7). Several hundred to thousands of cercaria are released per snail. In order to identify transgenic clones from among productive infections, aliquots of 100 cercaria are centrifuged and subjected to PCR analysis using primers specific for transgene sequences (Box 7, A). Southern blot analysis is performed on the positive clones in order to eliminate clones carrying the transgene as an extrachromosomal array, which is not expected to be stably maintained due to the lack of centromeres. (For analysis of this phenomenon in another helminth (*C. elegans*), see Stinchcomb DT et al., Extrachromosomal DNA Transformation of *Caenorhabditis Elegans*, Molecular and Cellular Biology 5:3484-96, 1985) Extrachromosomal arrays are identified by the absence of higher molecular weight bands that correspond to segments of schistosome genomic DNA flanking the integration site revealed by Southern blotting after digestion with a unique restriction site within the transgene, and by fast migration during electrophoresis of undigested DNA preparations (Box 7, A). Karyotyping of recombinant schistosome strains is performed on interphase chromosomes from cercaria. Males have homotypic (ZZ) sex chromosomes and are easily recognizable by a large heterochromatin fragment (Grossman AI et al., Sex heterochromatin in *Schistosoma mansoni*, Journal of Parasitology 66:368-70, 1980) (Box 7, B). Alternatively, PCR directed to the repetitive pW1 element can be used for *Schistosoma mansoni*, (Webster P, Mansour TE, Bieber D, Isolation of a female-specific highly repeated *Schistosoma mansoni* DNA probe and its use in an assay of cercarial sex, Molecular

and Biochemical Parasitology 36:217-22, 1989; Gasser RB, Morahan G and Mitchell GF, Sexing single larval stages of *Schistosoma mansoni* by polymerase chain reaction, Molecular and Biochemical Parasitology 47:255-58, 1991, both incorporated herein by reference) (Box 7, B).

Clonal populations of sporocysts are maintained by serial passage in snails in order to ensure preservation of primary lines during further characterization of adults, as described (Box 8) (Cohen LM and Eveland LK, *Schistosoma mansoni*: Long-term maintenance of clones by microsurgical transplantation of sporocysts, 1984; Chernin E, Transplantation of larval *Schistosoma mansoni* from infected to uninfected snails, The Journal of Parasitology 52:473-82, 1966, both incorporated herein by reference). Propagation by sporocyst transfer eliminates the need to follow the transgene segregation in further generations and permits salvage of potentially sterile schistosome clones. This method has been described in detail (Cohen LM and Eveland LK, 1984). Briefly, hepatopancreas and ovotestis are isolated from snails four weeks after onset of cercarial production, placed in Chernin's BSS (Chernin E, 1963), cut into narrow strips, and inserted through a puncture made with a 26 gauge needle into the cephalopodal sinuses of ten to twenty recipient snails. Cohen and Eveland reported transfer to 87% of surviving (56%) recipients. Thus, serial sporocyst transfer to ten snails should be sufficient to insure propagation of 99.9% of all clones in each round.

Repeat Southern blot analysis of transgenic clones is performed after three to five rounds of sporocyst transfer to confirm stable heritability of the transgene (Box 9). Adult worms are obtained in mixed bisexual infections of laboratory animals with transgenic cercarial clones and with wild type cercarial clones of the opposite sex (Box 10). Alternatively, cercariae are raised to adulthood in culture (Basch, 1981a and 1981b). Assay for release of desired protein into the host's bloodstream or culture medium is performed by an appropriate technique, such as ELISA, radioimmunoassay, or a specific bioassay or a chemical assay (Box 11). Pilot studies utilize human growth hormone as a reporter gene, which has a well-characterized and sensitive assay system (Selden RF et al.,

Molecular and Cellular Biology 6:3173-79, 1986). Immunohistochemistry and *in situ* hybridization are also performed in order to confirm the location of expression within the worms.

5 Breeding of transgenic clones (Box 12) may be necessary to ultimately obtain high-copy number clones of the appropriate sex. For clones secreting from the integument, male cercaria are desired for human subject infection and are easily obtained in bulk from the appropriate male transgenic
10 schistosome clones maintained in snails by sporocyst transfer. If the primary clone is female and fertile, then, to take advantage of this recombinant, a male transgenic clone must be selected from among the offspring of this clone after breeding with normal males by co-infection of the laboratory animal
15 host. Through additional crossbreeding of independent transgenic clones, progeny carrying multiple copies of the desired DNA construct can be identified, with a potentially dose-related increase in protein secretion. Likewise, for clones with expression targeted to female-specific secretory
20 structures, female transgenic cercaria must be similarly obtained.

III. Design of the Transgenic DNA Vector Construct

A. Local Promoter Elements

25 The injected transgene vector contains the cDNA sequence of the desired transgene within the DNA context required to direct a high level of tissue-specific expression, and the cDNA contains the signal sequences necessary to specify protein secretion. Figure 2A shows how genomic sequences from
30 a model schistosome gene (I) are used to produce the plasmid DNA construct containing the transgene (II). In this strategy, genomic sequences are incorporated *en bloc*. The upstream (a) and downstream (b) schistosome genomic fragments adopted to flank the transgene are each approximately 3.5 kb
35 long, to include local promoter and enhancer sequences. The model schistosome gene chosen has the desired pattern of tissue-specific expression in the integument, in the vitelline cells, in the Mehlis gland or in the ootype. To confirm that no other schistosomal genes are contained within these

flanking sequences, Northern blot analysis of schistosome RNA from all tissues and life cycle stages is performed using the flanking sequences as a probes, and open reading frames are found within the flanking sequences by DNA sequencing.

5 Also adopted into the transgene construct are 5' (c) and 3' (d) untranslated regions of the model gene, to promote proper post-transcriptional and post-translational processing within the schistosome target location. This includes trans-splicing (Rajkovic A et al., A spliced leader is present
10 on a subset of mRNAs from the human parasite *Schistosoma mansoni*, Proceedings of the National Academy of Sciences (USA) 87:8879-83, 1990), and intracellular trafficking to secretory pathways. The model gene coding sequence (e) is replaced by the transgene cDNA coding region (f). No intron is included
15 in the transgene transcript (g) since most schistosome genes are intronless. The amino-terminal signal peptide is derived from either the model gene or from the transgene. However, if the transgenic products are found to be expressed in schistosome cells but not secreted, then endogenous schistosome
20 (export) signal sequences, as putatively identified (Hawn TR, Tom TD and Strand M, Molecular Cloning and Expression of SmIRV1, a *Schistosoma mansoni* antigen with similarity to Calnexin, Calreticulin, and OvRall1, The Journal of Biological Chemistry, 268:7692-98, 1993, Chen L, Rekosh DM, LoVerde PT, *Schistosoma mansoni* p48 eggshell protein gene: characteriza-
25 tion, developmentally regulated expression and comparison to the p14 eggshell protein gene, Molecular and Biochemical Parasitology 52: 39-52, 1992) may be used in the construct exclusively. However, it is likely that these sequences show
30 enough evolutionary conservation to function heterologously.

As described below, further tailoring of the vector is performed to include higher order regulatory elements and to eliminate unnecessary sequences. The DNA vector is propagated within a bacterial plasmid. Plasmid sequences are not
35 microinjected into schistosome eggs and are removed from the transgene vector portion of the plasmid construct by restriction digestion at rare cutting endonuclease cloning sites (h, h') engineered into the plasmid. Following digestion at these sites, the DNA fragments are separated by

agarose gel electrophoresis or gel filtration and purified by standard techniques.

Appropriate schistosomal model genes are those of highly-expressed tissue-specific genes. For example, to target the
5 exogenous protein to the integument, the regulatory regions of tegumental antigen genes such as Sm15.9 (Abath FGC et al., Structure of the gene encoding a putative *Schistosoma mansoni* tegumental antigen precursor, Molecular and Biochemical Parasitology 60:81-92, 1993), Sm21.7 (Francis P and Bickle Q,
10 Cloning of a 21.7 kDa vaccine-dominant antigen gene of *Schistosoma mansoni* reveals an EF hand-like motif, Molecular and Biochemical Parasitology 50:215-24, 1992), Sm22.6 (Jeffs SA et al., Molecular cloning and characterisation of the 22-kilodalton adult *Schistosoma mansoni* antigen recognised by
15 antibodies from mice protectively vaccinated with isolated tegumental surface membranes, Molecular and Biochemical Parasitology 46:159-68, 1991), or of the glucose transporter genes, the SGTP's (Skelly PJ et al., Cloning characterization and functional expression of cDNAs encoding glucose trans-
20 porter proteins from the human parasite *Schistosoma mansoni*, The Journal of Biological Chemistry 269:4247-53, 1994) are used. To target to vitelline glands, flanking sequences from genes encoding eggshell proteins, such as p14 (Kunz WK et al., Sequences of two genomic fragments containing identical coding
25 region for a putative eggshell precursor protein of *Schistosoma mansoni*, Nucleic Acids Research 15:5894, 1987; Koster B et al., Identification of a putative eggshell precursor gene in the vitellarium of *Schistosoma mansoni*, Molecular and Biochemical Parasitology, 31:183-98, 1988) and
30 p48 (Chen L, Rekosh DM, LoVerde PT, 1992) are used. Targeting expression to the Mehlis gland and ootype is likewise performed with genomic sequences from Mehlis gland and ootype-specific genes, such as the those coding for Mehlis gland and ootype secretory products, cloned by tissue-specific differential expression or subtractive hybridization approaches.

Figure 2B shows how analysis of the model gene promoter/enhancer is used to increase the ability of the transgene to compete with the endogenous gene for transcription factors, thus reducing expression of the model gene while increasing

expression of the transgene. This approach is applied to the eggshell model gene in particular, to reduce production of granuloma-provoking eggs. The construct is graphically identical to the one in Figure 2A, except for the addition of tissue-specific core promoter elements (i). These sequences are identified in the model gene (I) promoter/enhancer region using standard DNase protection and gel shift analyses and by sequence analysis for sequence motifs conserved among promoters of different eggshell genes as well as among different species or strains. Then, a core element (i) (about 10 base pairs long), with its immediately-neighboring upstream and downstream sequences (about 80 base pairs long, in total) which likely contains binding sites for interacting transcription factors, is multimerized and reinserted into the promoter of the transgene expression construct (II) in its original location (e.g. in the proximal promoter) to increase tissue-specific expression of the transgene. It is also placed near the ends of the DNA construct to sop up tissue-specific transcription factors and reduce expression of the model (eggshell) gene.

The embodiment of this invention, depicted in Figure 2B (a transgene expression construct with extra tissue-specific core promoter elements), can be created without any preliminary experimentation. The vitelline gland eggshell gene p48 is the source of upstream and downstream promoter/enhancer regions. Based upon their presence in several similarly regulated vitelline-specific genes and upon evolutionary conservation to drosophila and silkworm eggshell genes, several putative core promoter elements have already been identified (Chen et al., 1992). One could use the 80 base pair region from -335 to -255 as the repeated segment (i). This sequence contains two putative core elements. One of these, "TCAGCT" (-278 to -273) is also found within the proximal promoters of the *S. masoni* p14 eggshell genes, as well as in silkworm and drosophila eggshell genes (for a complete discussion, see Chen et al 1992). In drosophila, this element has been shown to be essential for pattern-specific expression of the s36 chorion (eggshell) gene (Tolias PP, Konsolaki M, Halfon MS, Stroumbakis ND, and Kafatos FC,

Elements controlling follicular expression of the s36 chorion gene during *Drosophila* Oogenesis, Molecular and Cellular Biology 13:5898-5906, 1993).

B. Long-range Regulatory Sequences

5 Although flanking genomic sequences incorporated into the transgene vector are sometimes sufficient to target gene expression appropriately, "position effect" can alter the expression pattern of a transgene. That is to say, depending upon the site of integration into genomic DNA, the transgene
10 might be appropriately expressed, inappropriately expressed in undesired tissues, or not expressed at all. Usually, screening large numbers of recombinant organisms is sufficient to find a clone with the appropriate tissue-specific expression. Built-in higher order regulatory elements in the
15 transgene vector can reduce the relevance of the site of transgene integration into the schistosoma genome thus reducing the work to obtain the desired clones. Therefore, in one version of the transgene construct used in this invention, in addition to the nearby promoter and other flanking
20 regulatory elements of the model gene, two types of distant cis-acting elements derived from the model gene are incorporated into the vector.

 The first type is a locus control region (LCR). These regulatory segments, first described within the alpha- and
25 beta- globin loci, have now been discovered near several major gene clusters in vertebrates, including, for example, the recently described element near the T cell receptor alpha/delta locus (Diaz P, Cado D and Winoto A, A locus control region in the T cell receptor alpha/delta locus,
30 Immunity 1:207-217, 1994). Presumably, through topological alteration of a block of DNA (i.e. unwinding), locus control regions confer tissue-specific activation of large regions of chromatin, facilitating regulation of neighboring genes expressed at similar times in a particular tissue. They are
35 functionally defined as regions with a tissue-specific pattern of DNase hypersensitivity that, when incorporated into an expression construct, confer a tissue-specific, copy-number-dependent, integration-site-independent level of transgene expression (Grosveld R, von Assendelft GB, Greaves DR, and

George Kollias, Position-independent, high-level expression of the human beta-globin gene in transgenic mice, Cell 51:975-85, 1987). The *S. mansoni* p14 eggshell genes are clustered with two head-to-tail copies of one gene separated by a 7.5 kb region (Bobek LA, Rekosh DM, LoVerde PT, Small Gene Family encoding an eggshell (chorion) protein of the human parasite *Schistosoma mansoni*, Molecular and Cellular Biology 1988 8:3008-16, 1988), localized to chromosome 2 (Harai H, Tanaka M and LoVerde PT, *Schistosoma mansoni*: chromosomal localization of female-specific genes and a female-specific DNA element, Experimental Parasitology 76:175-81, 1993). It is likely that LCRs are important in their activation. LCR elements that regulate the model gene are identified based upon tissue-specific DNase hypersensitivity pattern (Tuan D and London IM, Mapping of DNase I-hypersensitive sites in the upstream DNA of human embryonic epsilon-globin gene in K562 leukemia cells, Proceedings of the National Academy of Sciences (USA) 81:2718-22, 1984; Tuan D et al., The "beta-like-globin" gene domain in human erythroid cells, Proceedings of the National Academy of Sciences (USA) 82:6384-88, 1985). These are then incorporated into the transgene construct to further refine and support transgene expression.

Analysis of the genomic structure of the model gene is required to find long range cis-acting LCR sequences. The search for these sequences begins at the proximal promoter of the model gene and proceeds in both the 5' and the 3' direction, to initially span up to 100kb of DNA in either direction. The search is confined to the region of the genome expressed in the tissue of interest, so each DNA segment is first used as a probe of Northern blots of schistosome RNA from the various tissues and life cycle stages. If, for example, a transcript that is expressed in the gut is identified 20 kb downstream of a model vitelline gland gene, then the LCR cannot lie beyond 20 kb downstream of the model gene. Thus, the region to be assayed for DNase hypersensitive sites can be delimited. To find DNase hypersensitive sites indicative of potential long range cis-acting LCR sequences, probes are generated near convenient restriction endonuclease sites spaced every 2 to 5kb, and DNase hypersensitivity assays

are performed as described (Tuan et al PNAS, 1985, Tuan and London, PNAS, 1984). For each putative hypersensitive site, control chromatin from a region of the worm not expressing the model gene or from the opposite sex (for female-specific transcripts) is used to determine if the hypersensitivity is tissue-specific. Non-tissue-specific DNase hypersensitivity sites are putative boundary elements and serve to delimit the region searched for long range cis-acting sequences. Figure 2C shows how a possible result of a search for an LCR is used to increase the activity of a transgene construct. In this hypothetical analysis, three upstream (j) and three downstream (k) tissue-specific DNase hypersensitive regions, each about 100bp, were identified. These sites are subcloned into the original construct (Figure 2A).

Construction of the transgene expression vectors is assisted by the polymerase chain reaction using primers containing restriction sites to facilitate cloning, if convenient sites are not present in the genomic DNA. Plasmid subcloning of genomic sequences, genomic mapping and DNA sequencing are performed according to standard techniques.

The other type of element to be incorporated into the vector is a boundary element. Boundary elements, recognized by their non-tissue-specific pattern of DNase hypersensitivity and by their ability to interact with specific protein factors (Zhao K, Hart CM and Laemmli UK, Visualization of chromosomal domains with boundary element-associated factor BEAF-32, Cell 81:879-89, 1995), are sequences scattered throughout the genome which isolate genomic units, preventing an activated genomic region from affecting neighboring transcription units (Kellum R and Schedl P, A position-effect assay for boundaries of higher order chromosomal domains, Cell 64:941-50, 1991). Boundary elements flanking the model gene are identified as non-tissue-specific DNA hypersensitivity regions or they are identified on the basis of ability to function as insulator elements in drosophila (Kellum and Schedl, 1991). Even copies of drosophila insulator/boundary elements (Udvardy A, Maine E, and Schedly P, The 87A7 chromomere: Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains, Journal of

Molecular Biology 185:341-358, 1985; Farkas G and Udvardy A .
Sequence of scs and scs' Drosophila DNA fragments with
boundary function in the control of gene expression, Nucleic
Acids Research 20:2604, 1992, incorporated herein by
5 reference) could be used for this purpose, as the sequences
display high evolutionary conservation, being functional in
distantly related species (Chung JH, Whiteley M and Felsenfeld
G, A 5' element of the chicken beta-globin domain serves as an
insulator in human erythroid cells and protects against
10 position effect in Drosophila, Cell 74:505-14 et al., 1993).
Therefore, one would expect the drosophila sequences to also
function in schistosomes.

In one version of the construct used in this invention
(Figure 2D), boundary elements (l)(from drosophila or
15 identified during the search for LCR sequences) are added to
the ends of the transgene vector depicted in Figure 2C outside
of the LCR. This step is to promote regulated transcriptional
control by preventing inappropriate activation of schistosome
genes near the site of transgene integration and by
20 functionally isolating the transgene from its surrounding host
chromatin.

Thus, with the boundary elements, LCR, and the proximal
promoter and flanking elements incorporated into the vector,
all of the sequences sufficient to confer integration-site-
25 independent, copy-number-dependent and tissue-specific
expression of the transgene are utilized. Not all of these
elements are incorporated into each version of the vector, and
simple vectors lacking distant LCR and boundary elements may
prove to function adequately when numerous recombinants can be
30 obtained. The identification of LCRs should not be considered
undue experimentation, although it may involve considerable
work, because the techniques involved (genomic DNA subcloning
and mapping, Northern blotting and DNase hypersensitivity
assays) are routine. Isolation of these regions is simply a
35 matter of iteration. The ultimate determinant of vector
adequacy rests upon assay of protein expression by transgenic
adult worms.

C. Additional considerations for vector design

The transgene vector construct can accommodate several kilobases of coding sequence with about 7 kb of flanking regulatory DNA. The length of the regulatory region of the transgene vector is minimized in order to save room for transgene coding regions and in order to maximize the number of molecules of DNA that can be injected into the egg, to increase the probability of recombination. Biochemical and functional assays are performed to obtain the smallest sufficient regulatory regions, retaining only minimally sufficient LCR and promoter elements. In biochemical assays the functional sequences of the regulatory cis-acting regions are highlighted by tissue-specific DNase hypersensitivity patterns. With sequential deletions of the initial construct, the minimally sufficient regions are further defined in vivo by testing their ability to regulate a reporter gene. For example, the cDNA for betagalactosidase could be incorporated into the vector, and the effectiveness of the regulatory sequences assessed by staining the adult worms with X-gal.

In addition, nuclease hypersensitivity studies require relatively pure cell preparations in order to identify the LCR and boundary elements. Vitelline gland cells are the predominant cell type in posterior female worm segments and in early eggs. Dissected tissue from these regions provides convenient source material for characterization of the eggshell genes. Identification of this region is facilitated by in situ fluorescent histochemistry (Bennet JL, Seed JL, Boff M, Fluorescent histochemical localization of phenol oxidase in female *S. mansoni*, Journal of Parasitology 64:941-44, 1978).

30

IV. Isolation of Model Genes

Genomic sequences corresponding to tissue-specific genes of interest are obtained from a schistosome genomic DNA library (lambda phage, cosmid or phage P1) using standard DNA hybridization techniques. Hybridization probes include cDNA and genomic DNA obtained from cooperating investigators, generated via PCR using oligonucleotides based on published sequences and obtained from schistosome libraries. Some of

the relevant cDNAs are identified by subtractive cDNA hybridization.

Identification of Mehlis gland-, ootype-, and vitellin cell-specific gene products by construction of subtracted

5 *cDNA libraries.* The sequences of several vitelline gland-specific cDNAs such as those encoding the p14 and p48 eggshell proteins are known and can be used as probes of genomic libraries to find the relevant regulatory sequences. However, in some embodiments of this invention the transgene

10 is expressed in the Mehlis gland and the ootype, sites which have yet to be assigned tissue-specific genes. It is not necessary to know the function of these gene products *per se*, since only the regulatory regions of these genes are used. A straightforward approach to finding these genes is subtractive

15 cDNA hybridization (Palazzolo MH et al., Use of a new strategy to isolate and characterize 436 drosophila cDNA clones corresponding to RNAs detected in adult heads but not embryos. Neuron 3:527-39, 1989; Sive HL, St John T, A simple subtractive technique employing photoactivatable biotin and

20 phenol extraction. Nucleic Acids Research 22:10937, 1988). In other embodiments of this invention, it is necessary to identify vitelline gland-specific transcription factors and tannase and phenolase enzymes. Here too, subtractive cDNA hybridization can be used to identify candidate genes that are

25 further subjected to sequence analysis, with putative identification based upon sequence homology to known genes. This process entails a considerable amount of work that is iterative in nature, but it has been performed successfully in many laboratories and does not require undue experimentation.

30 Subtractive cloning can be applied to find model genes. Whole female worm cDNA or cDNA from dissected female vitelline gland or Mehlis gland/ootype regions are depleted of male-expressed sequences by hybridization of female cDNA with photobio-tylated driver male RNA. To avoid isolation of

35 known and abundant female cDNA clones, *in vitro* transcribed known female-specific genes (e.g. p14 and p48 mRNA) are added to the driver population. A cDNA library is generated from this female-specific cDNA population. To characterize the spatial and temporal expression patterns of individual clones

in this library, Northern blot hybridization is used initially. Female worms are sectioned into anterior (Mehlis gland and ootype) region and posterior (vitelline gland) region, guided by *in situ* fluorescent histochemical identification of the vitelline gland region (Bennet et al, 1978). Also, mRNA from immature female worms from unisexual female infections and from eggs is isolated. The subtracted cDNAs are sorted by cross-hybridization pattern to identify clones of the same gene and by Northern blot hybridization to the various schistosome mRNA preparations mentioned. Probes from clones abundantly and specifically expressed in the vitelline gland, Mehlis gland and ootype regions are used for *in situ* hybridization to confirm the cell type of expression.

15 V. Interfering with Schistosome Females' Eggshell Production

For female worm clones with transgene expression targeted to the Mehlis gland, ootype or vitelline cells, interference with egg shell protein production or eggshell maturation is desirable to prevent provocation of a granulomatous response to viable eggs. Such shell-deficient eggs could be broken down by the host without provoking granuloma formation, as evidenced by the effect of vitamin C deficiency, which prevents eggshell hardening and granuloma formation in infected animals (Krakower C, Hoffman WA, Axtmayer JH, 1944). In general, in strategies targeting expression to female glands, two types of genetic modifications of the schistosome are required: the first one interferes with eggshell production, the second introduces the transgene. This sequential task is accomplished by performing the first modification in male worms (which become asymptomatic carriers) and the second one in their female, affected progeny.

Figure 3 shows a flowchart of the technique for creating transgenic schistosomes harboring a knockout construct for an eggshell gene or eggshell maturation enzyme. Female worms, paired with males in infected mouse intestines (A), lay stage

I eggs (B) *in vivo* or *in vitro*. As described above with regard to Figure 1, the stage I eggs are identified by a central fertilized egg that contains male and female pronuclei (i), surrounded by vitelline cells (ii) and a thin eggshell (iii). Both male and female stage I eggs are microinjected with a knockout DNA construct because they cannot be distinguished at the time of microinjection. This construct is designed to knock out an eggshell gene, an eggshell maturation enzyme (tyrosine hydroxylase or phenolase (see below)) or a vitelline specific transcription factor (see below). Karyotyping is performed on the clones at the sporocyst stage in infected snails (C), and female clones are discarded. Male cercaria (D) harboring the knockout construct are used to infect mice (E) in co-infections with wild type female cercaria. Normal female and male eggs harboring the eggshell knockout construct (F) are laid, because the vitelline cells are contributed by the wild type female parent. Both male and female eggs are now microinjected with the transgene construct, but this time female sporocyst clones (G) that have inherited the knockout construct and incorporated the transgene construct are retained. The cercaria (H) derived from these sporocysts are used with normal male cercaria to co-infect patients, producing viable adult worm pairs that lay soft eggs and produce large amounts of transgene product. Alternatively, transgenic female lines are obtained on a wild type strain background. If they are fertile, then they can simply be crossed with the male knockout construct-carrying line (D) by co-infection of mice, with selection of progeny female sporocyst clones harboring both the transgene and the knockout constructs.

RNA Knockout Vectors can be specifically designed. Antisense RNA is used naturally in diverse organisms to mediate destruction of complementary RNA strands, presumably by annealing with it and activating its digestion by RNases (reviewed by Delihias N, Regulation of gene expression by trans-encoded antisense RNAs, Molecular Microbiology 15:411-14, 1995). Hundreds of experiments have utilized this concept to down regulate specific messages, and the technique has proven highly useful for reducing biological activity of

- dozens of transcription factors in cell lines (e.g. Reis LF et al., Critical role of a common transcription factor, IRF-1, in the regulation of IFN-beta and IFN-inducible genes, EMBO Journal 11:185-93, 1992) and in transgenic animals (e.g.
- 5 Matsumoto K et al., Evaluation of an antisense RNA transgene for inhibiting growth hormone gene expression in transgenic rats, Developmental Genetics, 16:273-77, 1995) and plants (e.g. Kuipers AG et al., Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene expression
- 10 in potato, Molecular and General Genetics 246:745-55, 1995). Antisense constructs are generated by incorporating the cDNAs to targeted transcription factor, tanning enzyme or eggshell protein genes in reverse orientation into the expression construct, as described below.
- 15 Ribozyme constructs have also been shown to have biological utility, although it is unclear whether in practice, they are more effective than antisense RNA (James W and Al-Shamkhani A, RNA enzymes as tools for gene ablation, Current Opinion in Biotechnology, 1995 6:44-49, 1995).
- 20 Ribozyme expression constructs have been used in transgenic animals to knockout targeted gene function (Zhao JJ and Pick L, Generating loss-of-function phenotypes of the *fushi tarazu* gene with a targeted ribozyme in *Drosophila*, Nature 365:448-51, 1993). Ribozyme constructs are created with the
- 25 hammerhead ribozyme incorporated into short regions homologous to targeted schistosoma mRNA sequence, akin to antisense RNA constructs.

- Five strategies are described below for interfering with eggshell production: (1) Express the transgene at sufficiently
- 30 high levels such that it reduces eggshell expression by competing for factors essential for transcription, translation and protein processing of the eggshell gene products; (2) Use specific RNA-mediated knockout constructs (ribozyme or antisense) to destroy vitelline eggshell mRNAs; (3) Use
- 35 specific RNA-mediated knockout constructs to interfere with essential steps in eggshell maturation; (4) Interfere with transcription of differentiated vitelline cell-specific genes by RNA-mediated knockout of an essential vitelline cell transcription factor; and (5) Interfere with transcription of

differentiated vitelline cell-specific genes by expression of a dominant-negative or competitive inhibitor of an essential vitelline cell transcription factor.

In the first strategy, high level expression of the transgene in the vitelline cell directed by regulatory sequences of an eggshell gene may reduce tannable eggshell protein below levels needed to make functional tannable eggshells. Instead of releasing viable eggs, the gravid female releases the fertilized ova in a sea of transgene product. This high level expression is accomplished by the use of the most developed form of the vector, including boundary domain elements to flank the construct, and incorporation of relevant LCR-like and local enhancer and promoter elements to obtain position-independent, copy-number dependent expression, as outlined above in Figure 2D. The endogenous eggshell genes are present in the genome in multiple copies but they are not amplified as are the chorion genes of *Drosophila*. The p14 gene may only be present at about three copies per haploid genome of *S. mansoni* (Bobek et al, 1988 (see above)), and p48 is probably only present in a single copy, based on its low frequency in an unamplified genomic library (1 per 140,000 for p48 versus 6/100,000 for p14, Bobek, 1988, Chen, 1992 (see above)). At least two of the p14 genes are contiguous, separated by 7.5 kb of intergenic DNA, and arranged tail to head. Thus, if each transgene construct copy is transcribed at the same level as an endogenous p14 or p48 eggshell gene, then it would require 80 copies of the transgene construct to reduce eggshell protein production by 90%. This copy number is attainable by cross-breeding independent transgene integrants, considering that low transgene copy number clones are likely to remain fertile, because *ipso facto* they have preserved vitelline function. If an average transgenic schistosoma carries five tandem copies of the transgene, then four generations of cross-breeding three independent clones and selection of progeny achieves this goal.

Using the second strategy, RNA-mediated "knockout" constructs of eggshell genes are unlikely to significantly interfere with expression of these genes in the wild type

schistosome, since the target genes are expressed at high levels. However, expression of such knockout constructs may act synergistically with the above strategy to reduce already low eggshell gene transcripts to even lower levels.

5 The third strategy for interfering with eggshell production involves targeted interference with eggshell maturation. The rationale for this is that the schistosome egg shell requires enzymatic tanning (cross-linking) in order to harden. This outer shell is produced from secretory
10 products of the vitelline cells which envelop the egg in the worm uterus. Some of these cells adhere to the fertilized ovum and line the egg underneath the eggshell (LoVerde PT, and Chen L, Schistosome Female Reproductive Development, Parasitology Today 7:303-08, 1991). The main component of the
15 eggshell is sclerotin, a proteinaceous material that has undergone a quinone-dependent tanning process (Nollen PM, Digenetic trematodes: Quinone tanning system in eggshells, Experimental Parasitology, 30:64-67,1971; Wharton DA, The production and functional morphology of helminth eggs shells, Parasitology, 86 Suppl:85-97,1983; Smyth JD and Halton DW, The
20 physiology of Trematodes, 2nd Ed. Cambridge, Cambridge University Press, 1983) which cross-links and hardens the initially soft eggshell proteins. There is chemical evidence that the pathway involves phenols (i.e. dihydroxyphenylal-
25 nine) which are derived from modification of the numerous tyrosine residues that are present in the characterized eggshell proteins (Chen et al, 1992). These proteins are produced by the vitelline cells and released in vitelline granules along with an enzyme "phenolase" (Seed JL, Boff M and
30 Bennett JL, Phenol oxidase activity: Induction in female schistosomes by in vitro incubation, Journal of Parasitology 64:283-89, 1978, which oxidizes these residues to the highly reactive quinone. The quinones are thought to cross-link to exposed lysyl residues that are also present abundantly in the
35 eggshell protein molecules (Chen L. et al, 1992), hardening the shell of the schistosome egg.

The result of interfering with the eggshell tanning process is to prevent sclerotin hardening. The residual non-polymerized eggshell protein is not expected to provoke

disease, as mice treated with inhibitors of egg tanning do not develop hepatosplenomegaly (Bennett JL and Gianutsos G, Disulfuram: a compound that selectively induces abnormal egg production and lowers norepinephrine levels in *S. Mansoni*, Biochemical Pharmacology 27:817-20, 1978). An additional benefit is that for transgene products secreted by vitelline cells, softening the eggshell can be expected to facilitate diffusion of the transgene product into the host bloodstream instead of potentially becoming entrapped in the eggshell matrix.

Cloning eggshell maturation genes: The schistosome enzymes involved in this process have not yet been isolated. They are a putative tyrosine hydroxylase and a phenol oxidase. The tyrosine hydroxylase gene is to be cloned based on the extensive evolutionary sequence conservation. For example, the tyrosine hydroxylase genes of the fruit fly *Drosophila melanogaster* (Nechameyer WS and Quinn WG, Neuron 2:1167-75, 1989) and of the cow (Saadat S et al., J. Neurochemistry, 51:572-78, 1988) are 76% identical between *Drosophila* amino acid number 291 and 451. They are 71% identical in this region at the nucleotide level. Thus, the *Drosophila* sequence is used to probe at low stringency cDNA libraries made from female schistosomes or from early stage eggs, sources of vitelline mRNA. Alternatively, degenerate oligonucleotide primers based on the most conserved regions are used to generate an RT-PCR product for subsequent high stringency cDNA library screening (see Appendix).

A phenolase from *Drosophila melanogaster* involved in sclerotization of the exoskeleton has been cloned by genetic means and its sequence has been published (Pentz ES and Wright TRF, *Drosophila melanogaster* diphenol oxidase A2: gene structure and homology with the mast-cell tum(-) transplantation antigen, P91A Gene 103:239-42, 1991). Thus, low stringency screening of a female schistosome cDNA library may identify the clone of interest. In another strategy, expression cloning is used, with selection based upon enzymatic activity. A bacteriophage cDNA expression library is constructed (e.g. in Stratagene lambda Zap II). Induction of protein expression by IPTG is performed in the presence of 0.01M catechol and

assayed either on the agarose plates or on nitrocellulose filter lifts. The phenolase positive clones are identified by fluorescence at 537 nm when excited in the ultraviolet range (Bennet et al., 1978) or by the red color of the oxidation product (Wang FL et al., Isoenzymes of phenol oxidase in adult female *Schistosoma japonicum*, Molecular and Biochemical Parasitology 18:69-72, 1986). Alternatively, the phenolase and tyrosine hydroxylase are identified by subtractive hybridization, in which case schistosome tyrosine hydroxylase is recognized based upon predicted homology to other members of the family, and phenolase is recognized based upon homology to known oxidases. The cDNAs encoding both of these enzymes must show high level expression in the vitelline cells. Tyrosine hydroxylase is likely to be expressed in neurons as well.

A third potential target in this pathway is an activator of tyrosine hydroxylase, which has been found in mammals as well as drosophila, and has been cloned (Swanson KD and Ganguly R, Characterization of a *Drosophila melanogaster* gene similar to the mammalian genes encoding the tyrosine/tryptophan hydroxylase activator and protein, kinase C inhibitor proteins, Gene 113:183-90, 1992). It is not presently known if a similar activator is required for schistosome tyrosine hydroxylase activity. In vivo interference with the mRNAs encoding eggshell maturation enzymes is to be performed via antisense RNA or ribozyme constructs.

The fourth strategy for interfering with eggshell production involves blocking vitelline cell transcription factors. The most efficient way to reprogram vitelline cells to make the transgene product in place of eggshell proteins would be by manipulating expression of differentiated vitelline cell-specific transcription factors that coordinate expression of the gene products of differentiated vitelline cells. Typical transcription factors are expressed at levels orders of magnitude below many other gene products. In vitro and in vivo RNA-mediated knockout are most successful when the targeted moiety is expressed at low levels. (Cameron and Jennings, Antisense Res and Dev, 4:887-94, 1994) Therefore, RNA-mediated knockout strategies are expected to be more

successful when applied to these regulatory genes than when directed towards the gene products they regulate.

Vitelline gland maturation proceeds through four stages, with vitelline droplets appearing in the last stage (Erasmus DA, *Schistosoma mansoni*: development of the vitelline cell, its role in drug sequestration and changes induced by Astiban, Experimental Parasitology 38:240-56, 1975). The orderly pattern of cellular differentiation suggests the existence of stage-specific transcription factors to coordinate gene expression, with eggshell and tanning mRNAs expressed as a result of activation of the transcription factors of the terminally differentiated cell. The homologous promoter elements within the silkworm and drosophila chorion genes and the *Schistosoma mansoni* eggshell genes (Chen et al., 1992) are a starting point to clone candidate vitelline-specific, late-acting transcription factors. Cloning the factors that bind to those elements is readily achievable. Two methods have been routinely employed in this regard to clone dozens of DNA binding factors: (1) purification of the binding factor based upon affinity to synthetic oligonucleotides bearing the target sequence, followed by amino acid microsequencing and design of degenerate probes or PCR primers based upon reverse translation, and (2) direct probing of expression libraries with target element oligonucleotides. In addition, transcription factors can be identified on the basis of sequence homology after finding candidate tissue-specific genes by subtractive cDNA hybridization. This last method requires no prior information regarding specific target elements. After identification, these vitelline cell-specific transcription factors are to be targeted for interference by ribozyme and antisense constructs.

The fifth eggshell production interference strategy is dominant-negative interference with transcription factor activity. Current understanding of functional domains of many transcription factors as predicted their by primary amino acid sequence suggests a way to interfere with their function at the protein level. In general, the transcriptional activation domain is identifiable as a negatively charged amphipathic helix or a proline and/or serine/threonine rich domain. In

several cases, expression of a truncated version that lacks the essential transcriptional activation domain has been shown to inhibit the function of the endogenous wild-type factor by competition for target DNA elements (Langer SJ et al.,

5 Mitogenic signaling by colony-stimulating factor 1 and *ras* is suppressed by the *ets-2* DNA-binding domain and restored by *myc* overexpression, *Molecular and Cellular Biology* 12:5355-62, 1992; Lloyd A, Yancheva N and Wasylyk B, Transformation suppressor activity of a Jun transcription factor lacking its

10 activation domain, *Nature*, 352:635-38, 1991; Smith VM et al., GATA-3 dominant negative mutant, Functional redundancy of the T cell receptor alpha and beta enhancers, *Journal of Biological Chemistry*, 270:1515-20, 1995). Also, for transcription factors that bind to DNA as homodimers, overexpression of a

15 truncated form of the transcription factor lacking the DNA-binding portion of the protein has been shown to inhibit the function of the wild-type molecule in a dominant-negative fashion (Logeat F et al., Inhibition of transcription factors belonging to the rel/NF-kappa B family by a transdominant

20 negative mutant, *EMBO Journal* 10:1827-32, 1991; Beckman H and Kadesch T, The leucine zipper of TFE3 dictates helix-loop-helix dimerization specificity, *Genes and Development* 5:1057-66, 1991), similar to the naturally found Id family (Benezra R et al., The protein Id: a negative regulator of

25 helix-loop-helix DNA binding proteins, *Cell* 61:49-59, 1990) and to CHOP (Ron D, Habener JF, CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription, *Genes and Development*

30 6:439-53, 1992) factors. Most remarkably, for dimeric transcription factors containing leucine zipper motifs, simple rules have been derived based upon charge interaction to design effective competitive inhibitors (Vinson CR, Hai T and Boyd SM, Dimerization specificity of the leucine zipper-

35 containing bZIP motif on DNA binding: prediction and rational design, *Genes and Development* 7:1047-58).

It should be noted that knockout of a vitelline cell transcription factor complicates the strategy for transgene expression in strategies 4 and 5 above. To drive expression

in the transcriptionally crippled vitelline cells, the regulatory regions of the knockout construct are derived from a highly expressed, ubiquitous housekeeping gene. The promoter of such a gene does not require a vitelline cell-specific transcription factor for activity. Such a model gene could be one encoding a ubiquitous ion transporter (e.g. Na/K ATPase) or a glycolytic enzyme (e.g. triosephosphate isomerase (dos Reis MG et al., Characterization of the *Schistosoma mansoni* gene encoding the glycolytic enzyme triosephosphate isomerase, Molecular and Biochemical Parasitology 59:235-42, 1993)). The antisense RNA, ribozyme, or dominant negative knockout factor is active only in vitelline cells, where its target is present. The resulting vitelline cells, however, cannot be used to express the transgene under control of eggshell gene promoter elements. Thus, strategies 4 and 5 are feasible in conjunction with transgene expression in other tissues (e.g. the Mehlis gland and the ootype), where the vitelline gland specific transcription factor is irrelevant. This is illustrated in Figure 4A as a two-step strategy of schistosome genomic modification. In the first step (1) (see Figure 3, step B), eggs are microinjected with DNA from a plasmid construct containing a vitelline transcription factor knockout transcript (a) (i.e. ribozyme, antisense RNA, or dominant negative transcription factor). This transcript is under control of promoter/enhancer and LCR regions (b and b') taken from a housekeeping gene. In the second step (2) (see Figure 3, step F), a transgene whose expression is targeted to the Mehlis gland or ootype is microinjected, using DNA from a plasmid construct containing the transgene (c), flanked by regulatory promoter/enhancer and LCR sequences (d and d') taken from a Mehlis gland or ootype-specific model gene.

VI. Reprogramming Terminal Vitelline Cell Gene Expression by Introduction of a Replacement transcription Factor.

To take advantage of vitelline cell transgene targeting in conjunction with strategies to decrease eggshell gene transcription through interference with their specific transcription factors (strategies 4 and 5 above), a surrogate factor is designed to activate the transgene, and eggshell

protein expression is usurped by transgene expression. This is illustrated as a three step strategy of schistosome genomic modification in Figure 4B. In the first step (1) (see Figure 3, Step B), schistosome eggs are microinjected with DNA from a knockout construct encoding a transcript (a) targeting a vitelline gland tissue-specific transcription factor (TFA). The knockout constructs designed to antagonize TFA could be activated by non-tissue-specific "housekeeping gene" regulatory sequences (b and b' in construct 1a) or by tissue-specific regulatory sequences (constructs 1b and 1c). In the former case, the TFA knockout transcript (a) would be expressed in all cell types due to "housekeeping gene" promoter/enhancer sequences (b and b'), but it would be active only in mature vitelline cells, where it would encounter and inactivate TFA. In the latter case, tissue-specific expression of the TFA knockout RNA (a) could be achieved by utilizing the promoter context of TFA itself (c and c' in construct 1b), which would assure synchronous expression, but not high levels of expression. Alternatively (1c), tissue-specific expression of the TFA knockout RNA (a) in the context of regulatory sequences from a downstream eggshell gene (d and d') would enable high level expression of the knockout construct, but it would also allow transient expression of the eggshell genes.

In this three step strategy (Figure 4B), vitelline cell-specific expression of the transcript for the therapeutic transgene (h in construct 3) could not be facilitated simply by insertion within the context of eggshell gene regulatory sequences, as this would be inactive due to loss of TFA. Also, in the absence of TFA, the tissue-specific TFA knockout construct would cease to be transcribed (1b and 1c). To overcome this problem, the following scheme is employed: the cDNA for a transcript (e) encoding a replacement transcription factor B (TFB) is inserted into a construct in the second step (2) utilizing the vitelline cell-specific regulatory sequences of either TFA itself (c and c' in construct 2a) or of an eggshell gene (d and d' in construct 2b).

TFB is a chimeric protein consisting of TFA with its DNA-binding domain replaced by a DNA-binding domain with a

different and known specificity, choosing a replacement DNA-binding domain of the same class (e.g. basic, zinc finger, helix-turn-helix, homeobox.) However, if a dominant-negative knockout is used, only the transcriptional activation domain of TFA is incorporated into TFB, to avoid heterodimerization with the dominant-negative protein. As shown in the shaded inset, in the transgene expression vector (construct 3), all binding sites (e) for TFA within endogenous eggshell gene regulatory regions (exemplified by (f)) are functionally replaced by adding adjacent TFB binding sites (g). TFA sites within the regulatory region of the TFA knockout construct (1b and 1c) and of the TFB expression construct (2a, 2b) are likewise replaced by TFB binding sites, as indicated by the grey and the black boxes in elements (c) and (d) in Figure 4B. This step facilitates positive feedback regulation of the surrogate transcription factor TFB to mimic a likely aspect of TFA biology and stabilizes the TFA knockout expression. (This three step strategy is theoretically feasible for knockout of any terminal vitelline cell-specific transcription factor, barring the possibility that two or more necessary factors are both coordinately expressed and stabilize one another's expression. In that case it would be necessary to also replace the other factors, which could be done in the same expression construct as for TFB.)

This re-engineering of the vitelline cell gene regulation requires expression of two constructs (the TFA knockout construct and the TFB expression construct) in a male worm line to be transmitted as a dominant trait to progeny eggs to be used for subsequent microinjection of the therapeutic transgene. Co-injection of the TFA knockout and TFB expression constructs (in step B in Figure 3) is the simplest approach to obtaining this desired intermediate male clone. However, the TFB construct should be biologically silent even in females, so the triply transgenic lines can also be obtained by stepwise injection of the constructs into eggs of successive generations. This allows an intermediate step of crossing of TFB-carrying lines to titrate for optimal copy number. To wit, the TFB expression construct can be injected at step F (see Figure 3). Instead of discarding male

sporocyst clones at step G, the double transgenic males (harboring both the TFA knockout and the TFB expression construct) are mated with normal females by co-infection of mice (step E, Figure 3). In the final step, the transgene
5 construct is injected into the ensuing eggs, and female clones harboring all three constructs are selected for use.

It will be further apparent to one skilled in this art that the improvements provided for in the present invention, while described with relation to certain specific physical
10 embodiments also lend themselves to being applied in other physical arrangements not specifically provided for herein, which are nonetheless within the spirit and scope of the invention taught here.

**APPENDIX: PCR-CLONING OF SCHISTOSOME TYROSINE 3 HYDROXYLASE
GENE**

Oligonucleotides for PCR of tyrosine 3-hydroxylase gene from
5 vitelline cell cDNA:

Upstream primers (based on drosophila TY3H amino acids
368-373)

5' GA(T/C)GA(G/A)GA(G/A)ATTGA(G/A)AA(A/G)(T/C)T3'

5' GA(T/C)GA(G/A)GA(G/A)ATAGA(G/A)AA(A/G)(T/C)T3'

10 5' GA(T/C)GA(G/A)GA(G/A)ATCGA(G/A)AA(A/G)(T/C)T3'

Downstream primers (based on drosophila TY3H amino acids
428-433)

5' TG(A/G)TC(T/C)TG(G/A)TANGG(C/T)TGTAC3'

15 5' TG(A/G)TC(T/C)TG(G/A)TANGG(C/T)TGCAC3'

5' TG(A/G)TC(T/C)TG(G/A)TANGG(C/T)TGAAC3'

5' TG(A/G)TC(T/C)TG(G/A)TANGG(C/T)TGGAC3'

When combinations of upstream and downstream primers are used
20 in PCR reactions at annealing temperature of 50-60°C, a 195 bp
product is expected from vitelline cell RNA sources.

CLAIMS:

1. A method for producing transgenic schistosomes for secretion of desired gene products comprising:
 - a. identifying a gene whose expression is desired;
 - b. subcloning a protein-coding portion of a cDNA from said gene into a plasmid containing local upstream and downstream promoter/enhancer sequences, and containing 5' and 3' untranslated regions of a schistosome gene expressed in the integument, vitelline or Mehlis glands, or ootype of schistosomes;
 - c. microinjecting the transgene DNA into the pronuclei or cytoplasm of the zygotes of stage I schistosome eggs;
 - d. culturing said schistosome eggs to maturity in vitro;
 - e. allowing miracidia to hatch;
 - f. infecting snails on a one-miracidium-per-snail basis;
 - g. culturing said snails until cercaria of schistosome clones are produced;
 - h. identifying transgenic schistosome clones at the cercarial stage;
 - i. maintaining said transgenic schistosome clones by sporocyst transfer;
 - j. infecting a laboratory animal host with said cercaria of said transgenic schistosome clones on a one-clone-per-animal basis; and
 - k. identifying transgenic schistosome clones secreting high levels of transgene product into peripheral blood of said laboratory animal host.
2. The method of claim 1 wherein step (c) further comprises microinjecting the transgene DNA into the pronuclei or cytoplasm of the zygotes of stage I schistosome eggs laid in vitro.
3. The method of claim 1 wherein step (c) further comprises microinjecting the transgene DNA into the pronuclei or cytoplasm of the zygotes of stage I schistosome eggs dissected from tissues of an infected animal.
4. The method of claim 1 further comprising after step (h) the additional step of determining the gender of said transgenic schistosome clones at the cercarial stage, and wherein step (k) further comprises infecting a laboratory

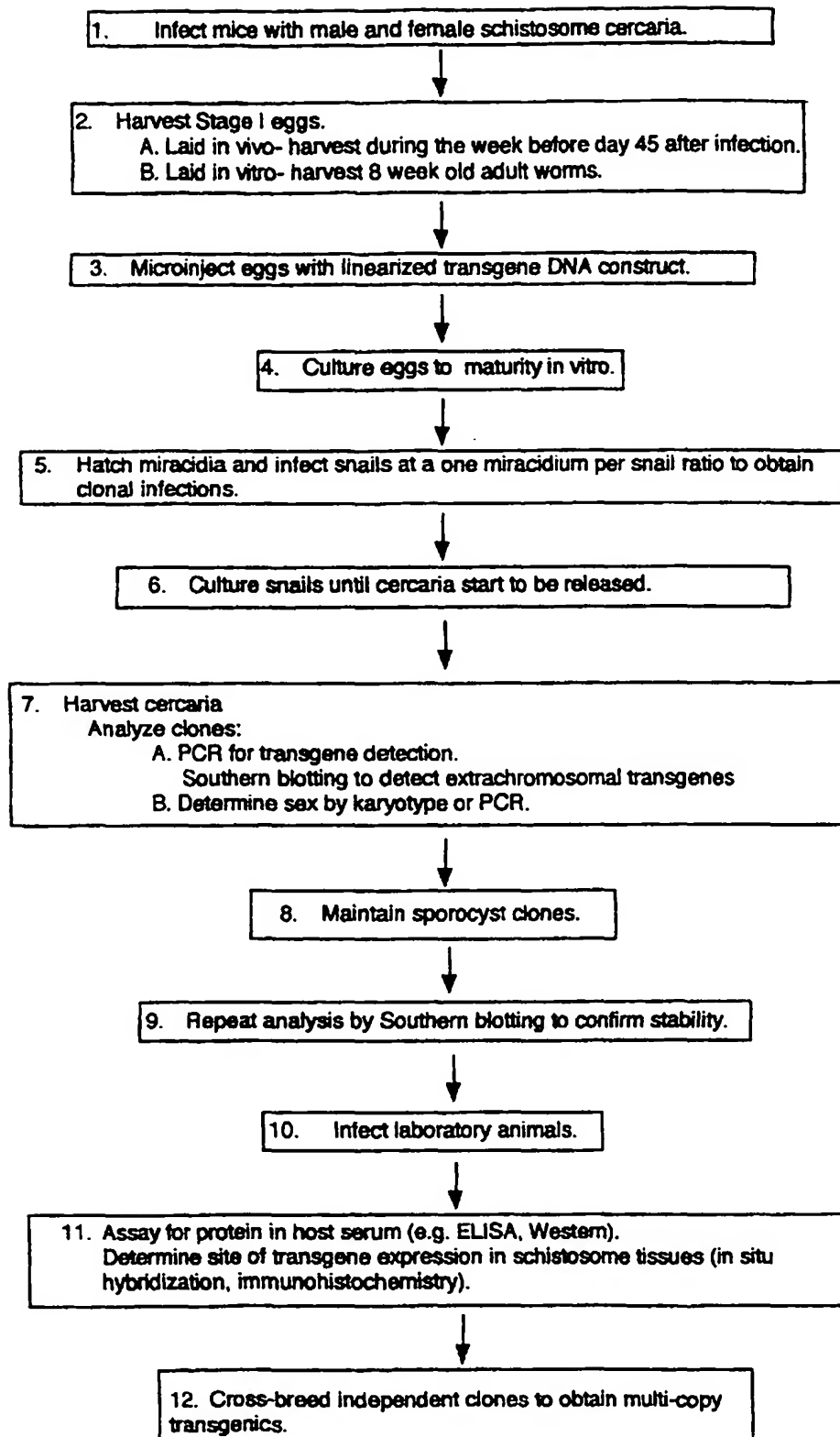
animal host with only male cercaria of said transgenic schistosome clones.

5. A method for producing therapeutic proteins in an animal host using transgenic schistosomes comprising:

- a. identifying a gene whose protein expression is desired;
- b. subcloning a protein-coding portion of a cDNA from said gene into a plasmid containing local upstream and downstream promoter/enhancer sequences, and containing 5' and 3' untranslated regions of a schistosome gene expressed in the integument, vitelline or Mehlis glands, or ootype of schistosomes;
- c. microinjecting the transgene DNA into the pronuclei or cytoplasm of the zygotes of stage I schistosome eggs;
- d. culturing said schistosome eggs to maturity in vitro;
- e. allowing miracidia to hatch;
- f. infecting snails on a one-miracidium-per-snail basis;
- g. culturing said snails until cercaria of schistosome clones are produced;
- h. identifying transgenic schistosome clones at the cercarial stage;
- i. maintaining said transgenic schistosome clones by sporocyst transfer;
- j. infecting a laboratory animal host with said cercaria of said transgenic schistosome clones on a one-clone-per-animal basis; and
- k. allowing said transgenic schistosome clones to secrete transgene product into peripheral blood of said laboratory animal host.

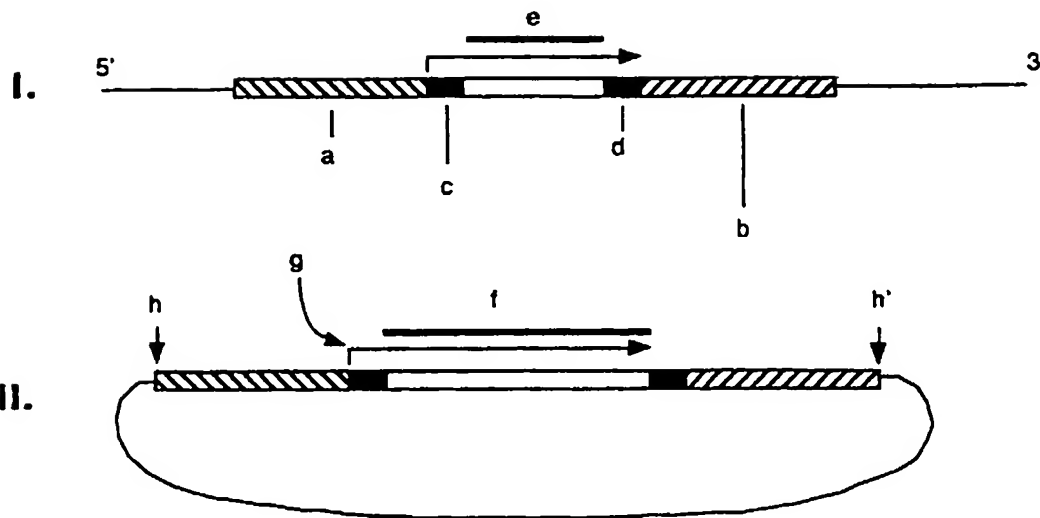
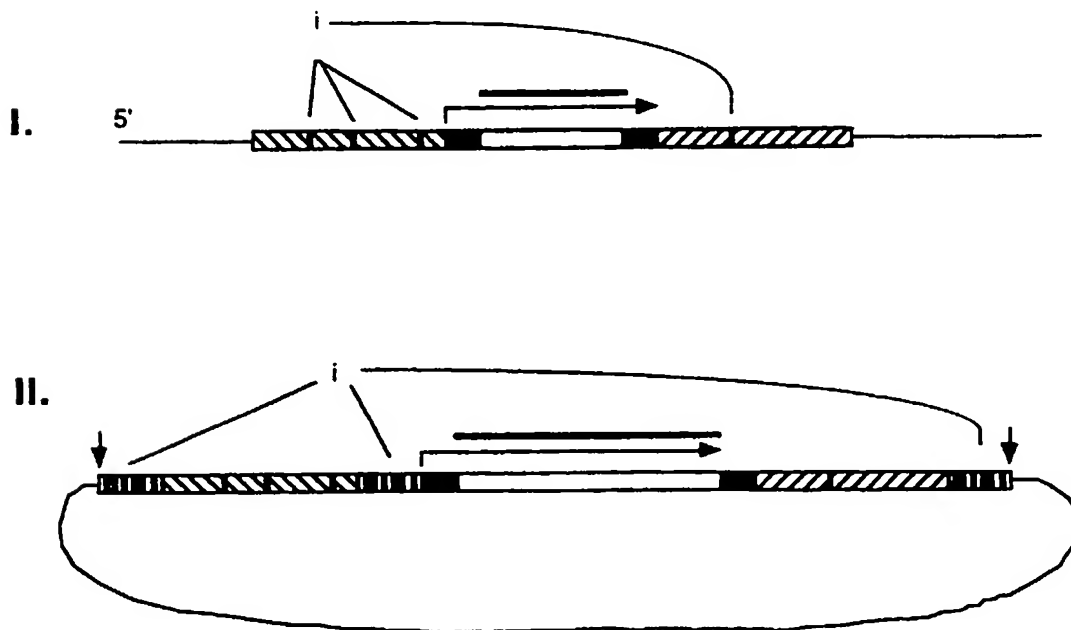
1/6

Figure 1

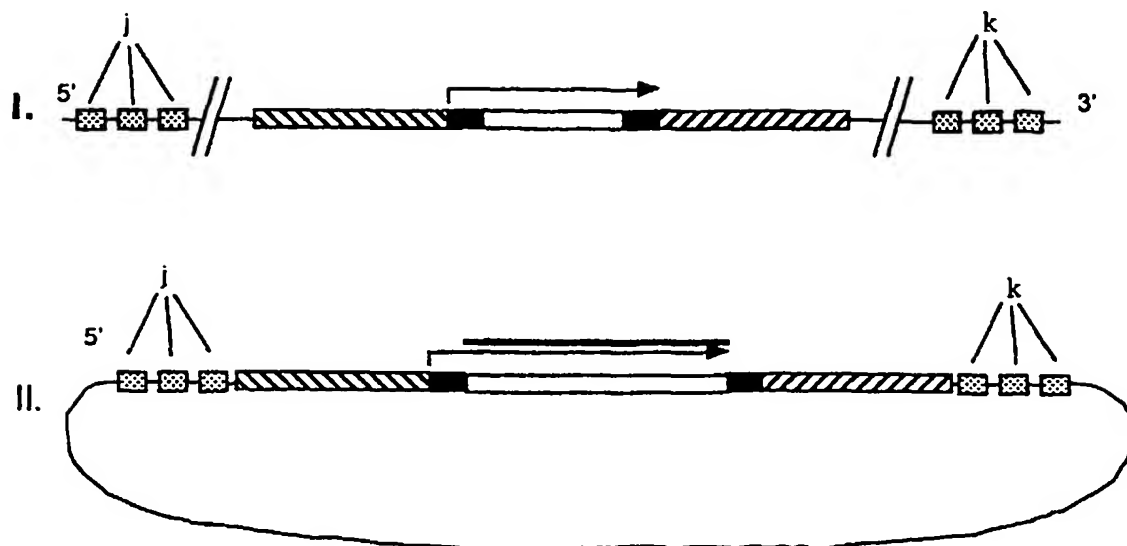


2/6

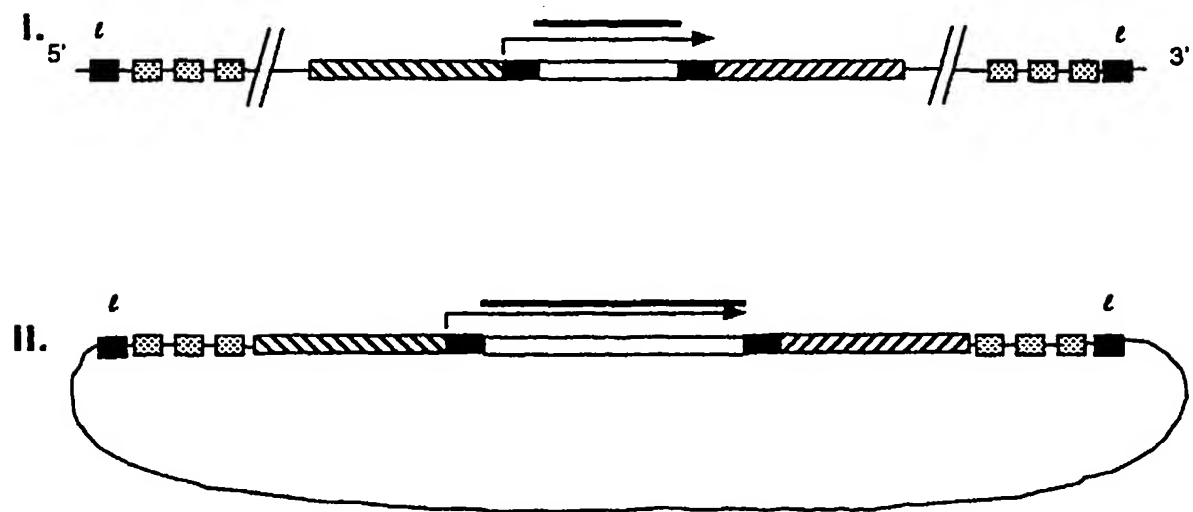
Figure 2.

A.**B.**

C.



D.



4/6

Figure 3

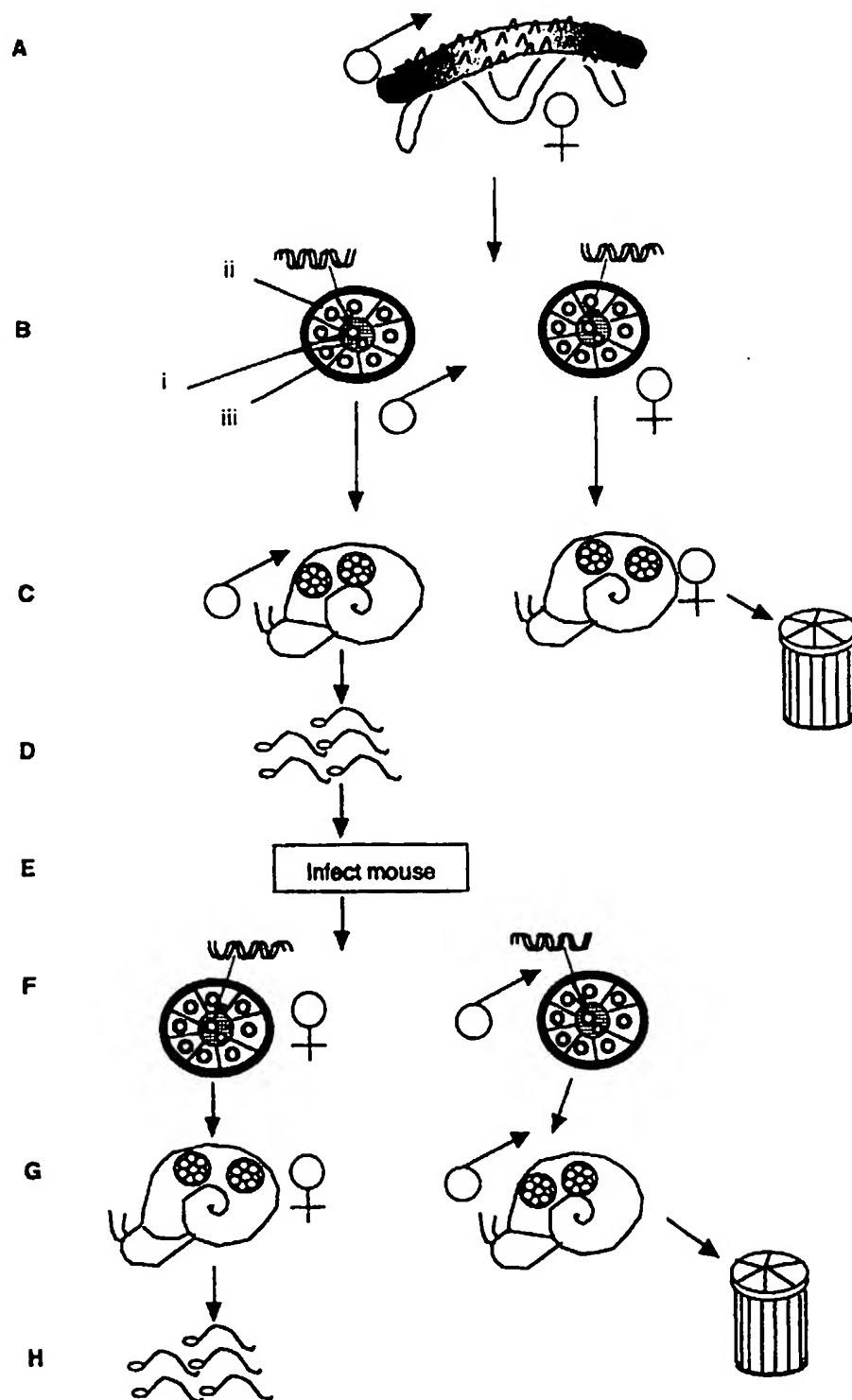
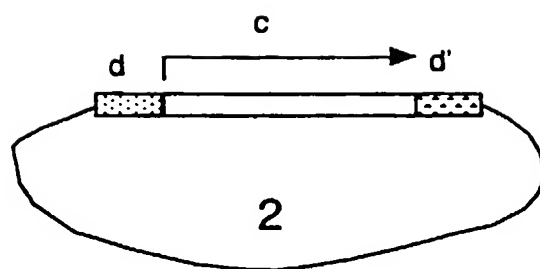
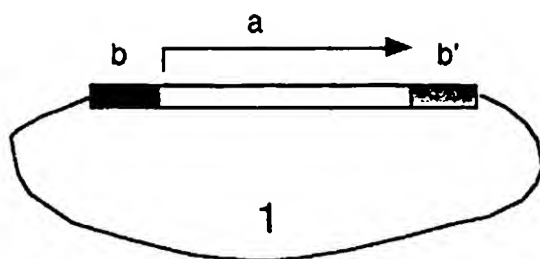
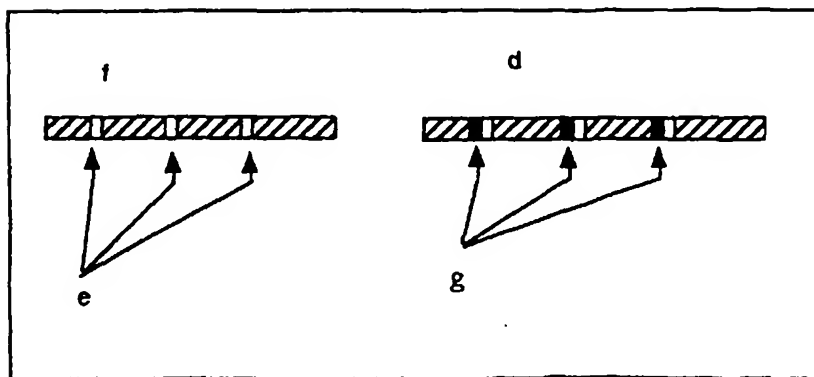
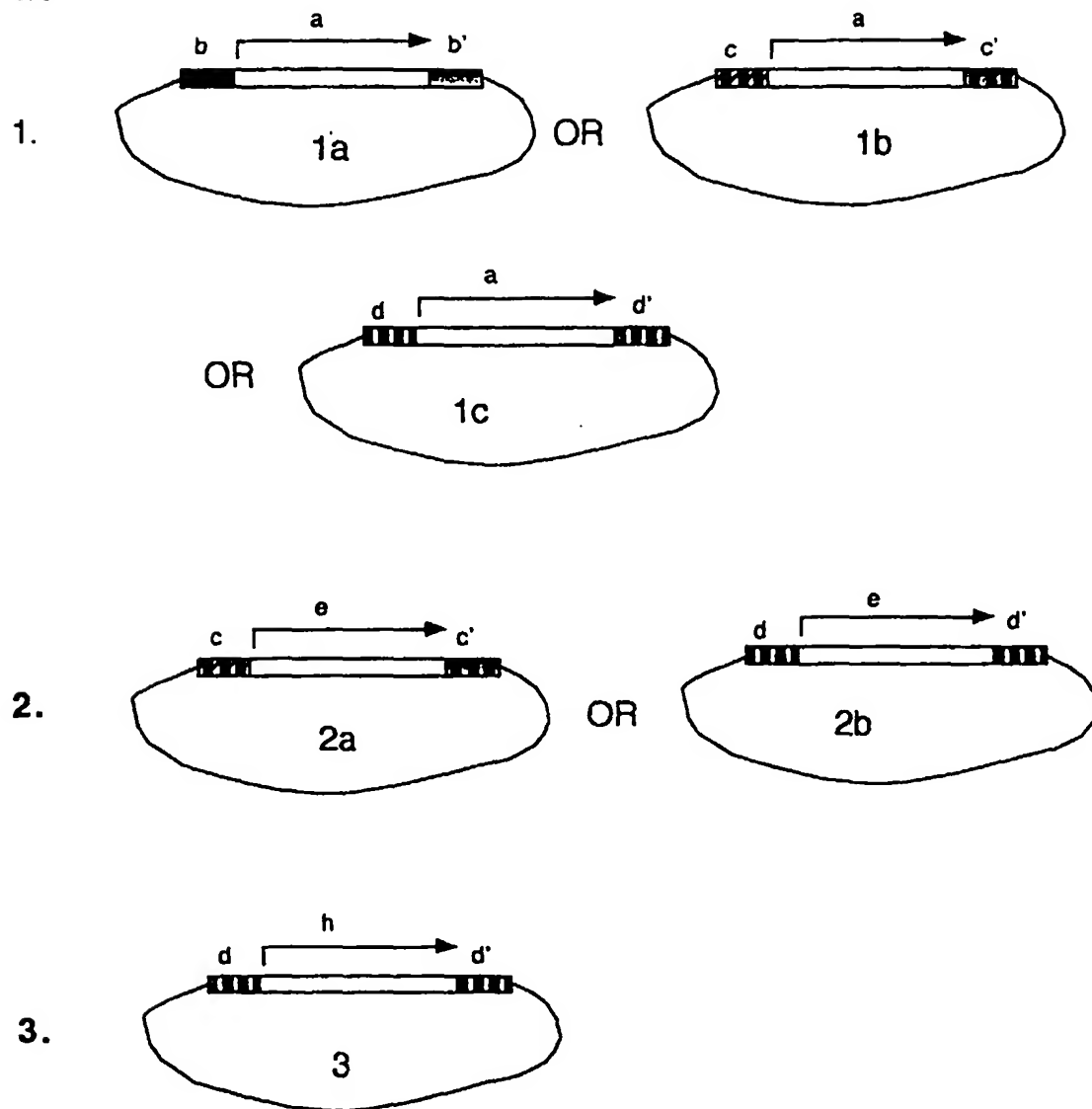


Figure 4.

A.



B.



INTERNATIONAL SEARCH REPORT

Inter. Application No
PCT/US 96/15083

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/89 C12N15/85 C12P21/00 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 554 064 (YEDA RES & DEV) 4 August 1993 see abstract -----	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

- * "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- * "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- * "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * "A" document member of the same patent family

Date of the actual completion of the international search

14 January 1997

Date of mailing of the international search report

30.01.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Hillenbrand, G

information on patent family members

PCT/US 96/15083

Form PCT ISA/210 (patent (priority annex) (July 1992))